Title: Transmission of antimicrobial resistant non-O157 *Escherichia coli* at the interface of animal-fresh produce in sustainable farming environments

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Abstract

The interaction of typical host adapted enteric bacterial pathogens with fresh produce grown in fields is complex. These interactions can be more pronounced in co-managed or sustainable farms where animal operations are, by design, close to fresh produce, and growers frequently move between the two production environments. The primary objectives of this study were to 1) determine the transmission of STEC or enteric pathogens from small and large animal herds or operations to fresh produce on sustainable farms in TN and NC, 2) identify the possible sources that impact transmission of AMR *E. coli*, specifically STEC on these systems, and 3) WGS to characterize recovered *E. coli* from these sources. Samples were collected from raw and composted manure, environment, and produce sources. The serotype, virulence, and genotypic resistance profile were determined using the assembled genome sequences sequenced by Illumina technology. Broth microdilution was used to determine the antimicrobial susceptibility of each isolate against a panel of fourteen antimicrobials. The prevalence of *E. coli* increased during the summer season for all sources tested. ParSNP trees generated demonstrated that the transmission of AMR *E. coli* is occurring between animal feeding operations and fresh produce. Ten isolates were identified as serotype O45, a serotype that is associated with the “Big Six” group that is frequently linked with foodborne outbreaks caused by non-O157 *E. coli*. However, these isolates did not possess the *stx* gene. The highest frequency of resistance was detected against streptomycin (n=225), ampicillin (n=190) and sulfisoxazole FIS (n=140). A total of 35 (13.7%) isolates from two TN farms were positive for the blaCMY (n=5) and blaTEM (n=32) genes. The results of this study show the potential of AMR *E. coli* transmission between animal feeding operations and fresh produce, and more studies are recommended to study this interaction and prevent dissemination in sustainable farming systems.

41 **Keywords:** Pathogen transmission, Sustainable production systems, Antimicrobial resistance,
42 Phylotyping, non-O157 *E. coli*

1. Introduction

According to the National Institute of Food and Agriculture (NIFA), sustainable farming systems operate under the premise of maximizing resource utilization by employing practices such as rotational grazing, nutrient management, or by limiting pesticide use (U.S. Department of Agriculture, National Institute of Food and Agriculture, 2018). As the general public becomes more aware of food safety, animal welfare, and farm sustainability, there has been an increase in the number of sustainable farms worldwide. However, practices like growing fresh produce near confined animal feeding operations (CAFO), applying raw and aged manure or inadequately composted teas or other biological soil amendments to fresh produce can increase the risk of the transmission of foodborne pathogens to fresh produce (Gutierrez-Rodriguez and Adhikari, 2018; Ingram and Millner, 2007).

The interaction of typical host enteric-adapted bacterial pathogens with field-grown fresh produce is complex and can occur due to multiple pathways including 1) the application of inadequately composted or raw as manure (Gutierrez-Rodriguez and Adhikari, 2018; Ingram and Millner, 2007; Tien et al., 2017); 2) surface water contamination via run-off from nearby CAFOs (Abakpa et al., 2015; Decol et al., 2017; Gutierrez-Rodriguez and Adhikari, 2018); 3) use of contaminated irrigation water or the use overhead irrigation instead of drip irrigation (Gutierrez-Rodriguez and Adhikari, 2018; Holvoet et al., 2013; Markland et al., 2013), and 4) pathogen transmission via insects and wildlife (Alves et al., 2018; Gutierrez-Rodriguez and Adhikari, 2018; Talley et al., 2009; Wasala et al., 2013). Foodborne pathogens like Shiga toxin producing *Escherichia coli* (STEC) O157:H7 and non-O157:H7 STEC has dominated the recent fresh produce-related outbreaks in the United States. In the past three years, multiple outbreaks both in quick service restaurants as well as grocery store products including alfalfa sprouts, leafy greens,

and romaine lettuce have been reported in the U.S. (U.S. Department of Health & Human Services, Centers for Disease Control and Prevention, 2016a, 2016b, 2018a, 2018b, 2019). Furthermore, analysis of whole genome sequencing (WGS) data generated from the 2018 romaine lettuce multistate outbreak ending in June 2018, identified resistance to several antimicrobials including ceftriaxone, a third-generation cephalosporin (U.S. Department of Health & Human Services, Centers for Disease Control and Prevention, 2018a). Antibiotic resistance (AMR) and multidrug-resistant (MDR) foodborne pathogens, which are resistant to at least three or more classes of antimicrobials, can also be isolated from environmental samples like manure (Abakpa et al., 2015; Marti et al., 2013; Tien et al., 2017), irrigation water (Abakpa et al., 2015; Faour-Klingbeil et al., 2016; Holvoet et al., 2013), fresh produce (Abakpa et al., 2015; Faour-Klingbeil et al., 2016; Holvoet et al., 2013; Ranjbar et al., 2016), soil (Abakpa et al., 2015; Holvoet et al., 2013; Tien et al., 2017), and insects (Alves et al., 2018; Mohammed et al., 2016; Ranjbar et al., 2016). These findings accentuate the heightened risk of outbreaks of AMR and MDR pathogens caused by contaminated fresh produce and the need for further understanding of the mechanisms of contamination involved at the farm level.

Although there have been some studies focused on identifying AMR foodborne pathogens isolated from fresh produce (Abakpa et al., 2015; Faour-Klingbeil et al., 2016; Holvoet et al., 2013; Reuland et al., 2014), few have studied the transmission of these pathogens from livestock to fresh produce (Abakpa et al., 2015; Muloi et al., 2018). Furthermore, there have been several studies focusing on the prevalence of pathogens in organic versus commercial farms (Gutierrez-Rodriguez and Adhikari, 2018; Kim and Woo, 2014; Leff and Fierer, 2013). However, a comprehensive study examining the transfer of these pathogens from livestock to fresh produce on operating sustainable farms is lacking, especially with concern to sustainable

farms where CAFOs are in very close proximity to fresh produce (Berry et al., 2015; Faour-Klingbeil et al., 2016). It is known that cattle are asymptomatic carriers of STEC, and a recent study in New Zealand discovered that 20% of cows were carriers of the O157, O26, O45, O103, O111, O121, and O145 serotypes (Browne et al., 2018). The latter six serotypes are the most prevalent non-O157 serotypes among human isolates in the U.S. and are referred to as the “Big Six” (U.S. Department of Agriculture, Food Safety and Inspection Service, 2012). One step to reducing the burden of disease due to the consumption of contaminated fresh produce is to identify the different sources of contamination and to develop mitigation practices that can reduce or eliminate the transfer of AMR foodborne pathogens to fruits and vegetables.

The main aims of this study were to determine if transmission of STEC or enteric pathogens from small and large animal herds or operations to fresh produce could occur in small sustainable farms, to identify the possible sources of AMR *E. coli* on these systems that rear livestock and grow fresh produce in proximity, and to use WGS to characterize recovered *E. coli* from these sources. Information collected from these types of studies could begin to provide initial insights into any potential transmission of enteric pathogens in small sustainable commercial farms, help identify sources and track movement, and list those conditions that favor pathogen transmission risk from small livestock operations to fresh produce.

2 Materials and Methods

2.1. Experimental design

A total of five commercial sustainable farming systems, two in North Carolina (NC; farms 1 and 2) and three in Tennessee (TN; farms 3-5), were sampled over two years between the months of May-September of 2014-2015. Farms in NC were small in scale (below 50 acres),

with farm 1 consisting of a mobile chicken coop containing between 30-50 birds and farm 2 rearing 5-10 dairy cattle at any one time. In comparison, TN farms 3-5 were large scale productions. Farm 3 (1,500+ acres) had 80,000 chickens 25 acres of fresh produce, while farm 4 (approximately 400 acres) had 120,000 chickens and 25 beef cattle. Finally, farm 5 grew fresh produce and reared cattle, pigs, sheep, and goats on 300 acres of land. None of the farms in this study were isolated from their surrounding communities. Produce samples were collected on these farms depending on the availability of produce, geographic location, and season. Fresh fecal and manure samples from livestock (poultry, cattle, swine, and small ruminants), fresh produce (leafy greens, tomatoes, melons, snap beans, and cucumbers), and environmental samples (flies, soil, and irrigation water) were collected within a 3 to 1,200 m distance from CAFOs or small animal herds. All the samples were transported back to the respective lab inside coolers with ice keeping all samples at or below 8 °C.

2.2. Produce Collection and Enrichment

A total of five 150 g replicates were collected per crop. In NC, all produce samples were collected within 30-40 m of the small animal herd. Crop rotation varied between NC and TN and growing season. In TN produce samples included snap beans, corn, cucumbers, melons, tomatoes, red and green lettuce, turnips, and squash; while in NC produce samples were mainly watermelons, peppers, melons, spinach, cabbage, tomatoes, and broccoli. Each sample was randomly selected and aseptically collected to avoid cross-contamination from adjacent crops or standing water. When harvesting tomatoes, different ripening stages (mature green [n=1], “turning” [n=2], and “red” [n=3]) were collected along the cropping cycle or sampling event looking to better represent the conditions of the plant and fruit through the growing season.

For STEC enrichment from fresh produce (tomatoes, leafy greens and (water)melon rinds), we followed a modified procedure from Gutierrez-Rodriguez et al. (2012, 2019). Briefly, a total of 300 mL of 2X Universal Pre-enrichment Broth (UPB) (Becton, Dickson and Company, Franklin Lakes, NJ) with 0.05% (v/v) Tween20 (Fisher Scientific, Waltham, MA) was added to 150 g of produce. After a one-minute massage, samples were sonicated for 30 min. The supernatant was then transferred to a new container. Following an 18-24 hour incubation at 35 °C, 10 mL of the pre-enrichment were transferred to 90 mL of mEHEC broth (MilliporeSigma, Burlington, MA) and incubated at 37 °C for 18-24 h. After enrichment, all samples were on CHROMagar STEC (CAS) (CHROMagar Microbiology, Paris, France) amended with 25% of the amount supplements instead of 100% to account for genetic variability and different resistant levels from naturally occurring STEC in different farming environments and environmental samples. Plates were incubated at 35 °C for 24-48 h. Single isolated mauve colonies from CAS were cleaned three times using CAS and tryptic soy agar (TSA) (Becton, Dickson and Company, Franklin Lakes, NJ) and was then suspended in potassium phosphate buffer and stored in 26% (v/v) glycerol. In some instances when dealing with fastidious environmental strains, isolated colonies from CAS were struck on Luria-Bertani (LB) (Becton, Dickson and Company, Franklin Lakes, NJ) agar and incubated at 37 °C for 18-24 h. The isolates were then stored in LB broth with 20% (v/v) glycerol (Becton, Dickson and Company, Franklin Lakes, NJ) at -80°C until further characterization. This enrichment process was repeated for leafy greens and watermelon rinds.

2.3. Manure Collection and Enrichment

During each visit, 150 g of raw and composted manure was collected from livestock (dairy, poultry, and small ruminants) from each farm. Care was taken to collect from fresh manure piles with sterile plastic scoops and to avoid the interface with the ground. For NC farm 1, raw chicken manure was collected from five locations within the mobile chicken coop while in farm 2, raw dairy manure was collected from five different locations within the pasture. Raw chicken manure from TN farms 3 and 4 was collected from three locations (front, middle, and back) within each of the four chicken houses. Raw manure from cattle, pigs, goats, and sheep from TN farm 5 was collected from three locations within each pen.

During processing, 25 g of manure was combined with 225 mL of 1X UPB and massaged for 1 min. The supernatant was then transferred to another container and incubated at 35°C for 18-24 h. The next day, 10 mL of the pre-enrichment was transferred to 90 mL of mEHEC broth and incubated at 37 °C for 18-24 h. After enrichment, all samples were struck on CAS amended with 25% (v/v) supplements. Plates were incubated at 35 °C for 18-24 h. Single mauve colonies were isolated in a similar way as described previously.

2.4. Farm Environment Samples

During each visit water, soil, and flies were collected in NC and TN samples. In NC water sampling was performed once each year and consisted of collecting three 3.8 L samples at the outlet of the irrigation system within farm 1 (poultry) and farm 2 (dairy) operations. In TN water sampling was performed monthly from July to September and consisted of collecting one 3.8 L sample from each water source. Water was analyzed following the procedure described by Sbodio et al. (2013) for generic *E. coli*. In brief, each one-gallon water sample was filtered

through a modified Moore swab system, and the *E. coli* population was determined by plating on CHROMagar ECC (CHROMagar Microbiology, Paris, France) agar.

Soil samples were collected from predetermined distances within each farm in NC and TN. In NC, these twenty soil collection sites on each farm were selected and marked with flags. Sampling was performed in a Z pattern using a sterile 113 g scoop and soil was collected from the top 15 cm of the soil profile. Up to 20 samples were pooled (500 g) in order to get a representative sample of the prevalence of STEC in soil due to the small nature of the plots (30-40 m) within each farm (Gutierrez-Rodriguez et al., 2012). Each pooled sample was mixed with a sterile scoop and from the mixed sample, 150 g was used for the isolation of STEC. In brief, 150 g of soil was combined with 300 mL of (0.02M) sodium phosphate with 0.05% (v/v) Tween20 and was massaged for 1 min. The supernatant was then transferred to a new sterile bag containing 250 mL of 2X UPB and incubated at 35 °C for 18-24 h. The next day, 10 mL of the pre-enrichment was transferred to 90 mL of mEHEC broth and incubated at 37 °C for 18-24 h. After enrichment, all samples were streaked on CAS amended with 25% supplements (Gutierrez-Rodriguez et al., 2012). Plates were incubated at 35 °C for 18-24 h. Single mauve colonies were isolated in a similar way as described previously.

A live sampling of the fly population was performed using nets at each sampling site (soil, livestock, irrigation water, and produce) during each farm visit of all TN farms and NC farm 1. Four preselected locations (manure, vegetables, fruit, and water) at every farm site was outfitted with five QuikStrike fly strips (Wellmark International, Schaumburg, IL) with attractant and insecticide that kills flies on contact. Care was taken to avoid direct sun and rainfall contact during placement of the fly strips. At the lab, the species of each insect was identified and quantified before sorting representative flies into two groups (house flies and blowflies) of 5-10

insects. The experimental procedure followed for *E. coli* was based on the procedures described previously by Olsen and Hammack (2000). In brief, fly samples totaling no more than 1 g were pre-enriched in 10 mL of 2X UPB for 18-24 h at 35 °C. The next day, 10 mL of the pre-enrichment was transferred to 90 mL of mEHEC broth and incubated at 37 °C for 18-24 h. After enrichment, all samples were streaked on CAS amended with 25% supplements (Gutierrez-Rodriguez et al., 2012). Plates were incubated at 35 °C for 18-24 h. Single mauve colonies were isolated in a similar way as described previously.

2.5. Antibiotic Susceptibility Testing

Broth microdilution was used to characterize the phenotypic AMR profile of the *E. coli* isolates from both TN and NC and to determine the minimum inhibitory concentration (MIC) as described by Pornsukarom, Suchawan et al. (2018; http://www.uniscience.co.kr/data/trds/sensi_manuals/VET_Mic_panel.pdf). The Sensititre gram-negative National Antimicrobial Resistance Monitoring System (NARMS) CMV3AGNF plate (Trek Diagnostic Systems, Cleveland, OH), was used to complete this assessment. The isolates were exposed to a panel of fourteen preselected drugs: amoxicillin/clavulanic acid (AUG2; 1/0.5–32/16 µg/mL), ampicillin (AMP; 1–32 µg/mL), azithromycin (AZI; 0.12–16 µg/mL), cefoxitin (FOX; 0.5–32 µg/mL), ceftiofur (XNL; 0.12–8 µg/mL), ceftriaxone (AXO; 0.25–64 µg/mL), chloramphenicol (CHL; 2–32 µg/mL), ciprofloxacin (CIP; 0.015–4 µg/mL), gentamicin (GEN; 0.25–16 µg/mL), nalidixic acid (NAL; 0.5–32 µg/mL), streptomycin (STR; 32–64 µg/mL), sulfisoxazole (FIS; 16–256 µg/mL), trimethoprim/sulfamethoxazole (SXT; 0.12/2.38–4/76 µg/mL), and tetracycline (TET; 4–32 µg/mL). The breakpoints were determined by the standards set by the Clinical and Laboratory Standards Institute, and the National Antimicrobial

Resistance Monitoring System (NARMS) was used to determine the breakpoints (Clinical and Laboratory Standards Institute, 2013, 2015; U.S. Food and Drug Administration, National antimicrobial Resistance Monitoring System, 2012). An isolate was designated as MDR if it exhibited resistance to three or more class of antimicrobials.

2.6. DNA Isolation and Whole Genome Sequencing

A modified version of the Qiagen DNeasy Blood & Tissue kit (Qiagen, Venlo, Netherlands) was used to isolate the DNA of the TN and NC *E. coli* isolates. During cell lysis, the samples were incubated for 3 h and vortexed every 20 min for 10 s. 4 µL of RNase A was added to each sample in order to remove RNA contamination. Samples were centrifuged at 10,000 rpm during the final wash steps. A pre-warmed 10 mM Tris-HCl (pH 8.0) (Fisher Scientific, Waltham, MA) solution was used as the elution buffer for each DNA sample. The high-sensitivity assay kit for the Qubit 4.0 Fluorometer was used to verify the concentration of double-stranded DNA (ThermoFisher Scientific, Waltham, MA). Once quantified, DNA libraries of each sample were prepared for sequencing using a Nextera XT kit (Illumina, San Diego, CA). Multiplexed sequencing of these libraries was done with a single run on an Illumina MiSeq (Illumina, San Diego, CA) using 250 bp or 300 bp paired-end reads (MiSeq reagent kit, version 3). Samples were de-multiplexed and submitted to the National Center for Biotechnology Information GenomeTrakr database (Accession: PRJNA293225).

2.7. Genome Sequence Analysis

Serotype characterization of the O- and H-antigens was performed using the SerotypeFinder (Joensen et al., 2015) and EcOH (Ingle et al., 2016) databases for all

presumptive STEC isolates. The program ABRicate (<https://github.com/tseemann/abricate>) was then used to screen for pathotypes, extended-spectrum β -lactamases (ESBL), and AMR/virulence genes using the Resfinder (Zankari et al., 2012), CARD (Jia et al., 2017), ARG-ANNOT (Gupta et al., 2014), NCBI BioProject PRJNA313047 (<https://www.ncbi.nlm.nih.gov/bioproject/>), and VFDB for *Escherichia* (<http://www.mgc.ac.cn/VFs/main.htm>) databases. *E. coli* pathotype categories were determined using the guidelines suggested by Robins-Browne et al. (2016). *E. coli* phylogroups (A, B1, B2, C, D, E, F and G) for the TN and NC *E. coli* isolates were determined using an *in silico* quadruplex PCR method (Beghain et al., 2018; Clermont et al., 2019). Isolates were classified based on the presence or absence of four housekeeping genes: *arpA*, *chuA*, *yjaA*, and TSPE4 (Beghain et al., 2018). Phylogenetic trees were generated using the techniques described in Pornsukarom, S. et al. (2018). In brief, the tool ParSNP from the Harvest suite (Treangen et al., 2014) was used to identify single-nucleotide polymorphisms (SNP) present in the chromosome of each isolate to determine how closely related each isolate is to one another. In order to get higher resolution mapping the settings were set to “-a 13” and “-x” (Van Vliet and Kusters, 2015). The PhiPack module was used in order to exclude SNPs located in known recombination regions of the genome (Pornsukarom et al., 2018). Phylogenetic trees were annotated and visualized using the programs Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>) and Treegraph v2 (Stover and Muller, 2010). The experimental design is depicted in figure 1.

3. Results

3.1. Prevalence of *E. coli* on NC and TN Farms

A total of 1659 samples comprised of produce (n=523), soil (n=329), raw manure (n=277), composted manure (n=70), poultry litter (n=178), water (n=55), and insects (n=227)

were collected from the five farms in NC (farm 1 and 2) and TN (farms 3, 4, and 5) during the experiment (Table 1). The number collected from each source varied slightly between each farm and with each visit. This is partially due to the farm management and weather patterns. The collective prevalence of *E. coli* for all sources tested was 15.4% (n=255), with 216 isolates coming from TN farms and 39 coming from NC farms. The majority of isolates were recovered from TN farms 3 and 4 (Figure 2), while farms 2 (NC) and 5 (TN) had the least (Figure 2). Although all sources (i.e., raw and composted manure, water, produce, and fly traps) tested were positive for *E. coli*, the main sources of *E. coli* were raw manure (46%), litter (24%), and insect (12%) samples collectively. When examined individually, the main sources of *E. coli* were raw manure (8%), insect (13%), and soil (8%) samples from NC farms, while the main sources in TN were raw manure (40%), litter (28%), and insect (12%) samples. TN farms 3 and 4 were the only farms to have all sources test positive for *E. coli* (Figure 2). Raw manure was the only source that tested positive from samples collected from NC farm 2 (Figure 2).

Of the 14 *E. coli* isolates that originated from fresh produce samples, the majority were isolated from cucumbers (n=6) sourced from TN farms 4 and 5 and snap beans (n=3) harvested from TN farm 3 (Figure 3). Interestingly, cucumbers were the only produce samples to test positive for *E. coli* in both 2014 and 2015, although these incidents occurred on different farms during the summer season. In contrast, snap beans (n=3) from TN farm 3 only tested positive in the summer of 2014 (Figure 3). Overall, several *E. coli* isolates came from sources (insect, water, raw chicken and cattle manure) that were located between 2655 to 3219 m away from the cucumber field on TN farms 3 and 4. Produce harvested from NC farms 1 and 2 never tested positive for *E. coli*.

The prevalence of *E. coli* increased between June and September for all farms in TN and NC farm 1 (Figure 4). Specifically, the prevalence of *E. coli* recovered from raw manure, litter, and produce sources increased during the summer months and peaked overall in July (Figure 4). This increase in prevalence in raw manure can be seen in both NC and TN farms 1, 3, 4, and 5, while only TN farms experienced an increase from produce (Figure 4). NC farm 2 experienced a spike in *E. coli* prevalence in manure samples in September and December, while TN farm 5 started to spike in May (Figure 4). No *E. coli* isolates were retrieved during January, February, April, October, and November (Figure 4). The month of June had the highest number of recovered *E. coli* isolates from TN farm 3 (n=53/93) followed by July (n=45/92) (Figure 4). The majority of these isolates were sourced from raw manure (June (n=17/53); July, (n=19/45)) and poultry litter (June (n=24/53); July (n=12/45)) samples (Figure 4).

3.2. Phenotypic AMR Profile

Based on the output from the broth microdilution testing, 159 out of the 255 presumptive STEC isolates were MDR and 23 were susceptible to all screened antimicrobials. Forty-two unique AMR resistant profiles were identified. Antibiotics STR (n=225/255), AMP (n=190/255), FIS (n=140/255), and TET (n=100/255) were the most prevalent phenotypic resistances in both states (Figure 5). Gen (n=98/255) was highly prevalent in TN (n=97/98) but not in NC (n=1/98) (Figure 5). The four most common AMR profiles were: AMP STR (n=48/255), AMP FIS GEN STR TET (n=29/255), AMP FIS STR (n=18/255), and AMP FIS STR TET (n=18/255). Resistance to CIP (n=1) was only found in TN and not NC (Figure 5). Resistance to AUG2, AXO, FOX, XNL, CHL, and AZL was detected in higher proportions in NC but not in TN.

3.3. ParSNP tree and phylogroups

As shown in figure 6, all eight *E. coli* phylogroups were identified using the in-silico PCR method (Beghain et al., 2018). Approximately, one-third of the isolates analyzed were determined to belong to phylogroups B2, D, E, F and G with the majority of isolates being classified as a member of phylogroups A and B1. A ParSNP tree was generated to compare the phylogenetic relationship of the core genome of the isolates. The tree revealed that there are two distinct branches that separate phylogroups A, B1, C, and E from phylogroups B2, D, F and G. Phylogroup F, which has recently split away from phylogroup B2, was the smallest group (n=3/255) categorized. Twelve of the *E. coli* was sorted into the newly identified phylogroup G. Interestingly, there was a few discrepancies with the phylogroup between the PCR method and the ParSNP tree (Figure 6). For example, isolates 91 and 242 are categorized as phylogroup A using the PCR method, but is clustered with phylogroup B1 using genomic data due to the loss of TspE4. In addition isolates 87 and 120 fall outside of the genomic clusters in the ParSNP tree but have been categorized as phylogroup B1 by the PCR method. Raw manure had the highest number of isolates in phylotypes A, B1, and D (n=38/117, 46/117, and 14/117, respectively) (Figure 7). Interestingly, 33% of insect samples were identified as phylotype D (Figure 7).

3.4. *E. coli* Serotyping

Out of 255 isolates, 70 different O-antigen serotypes were characterized using WGS. None of the isolates were identified as serotype O157:H7. The three most prevalent serotypes were O102, O12, and O45 (Figure 6). Of serotypes associated with the “Big Six”, only serotype O45:H8 was identified in ten isolates from TN (n=9) and NC (n=1). These isolates were determined to be a part of phylogroup B1 and did not possess the *stx* gene (Figure 6).

Interestingly, of the 14 positive produce samples, nine *E. coli* isolates from TN farm 3 and 5 belonged to serotype O45 (n=2) and serotype O102 (n=7). Isolates serotyped as O176, O138, and On12 were grouped into phylogroups C, E, and A (Figure 6). According to the phylogenetic tree, serotype O15 tends to cluster with phylogroup D (Figure 6). Interestingly, isolates serotyped as O102 were determined to belong to either phylogroups B1 or D (Figure 6). We were unable to identify the O-antigen of 25 isolates using the approach described above because there was not a direct match in the SerotypeFinder and EcOH databases.

3.5. Genotypic AMR Profile

WGS characterization of the isolates revealed that 141 isolates possessed resistance genes that can confer resistance to at least three classes of antimicrobials. Further characterization revealed the presence of 35 ESBLs *bla*_{CMY} (n=6/36) and *bla*_{TEM} (n=32/36), of which 82.9% were MDR. ESBLs were only recovered from TN farms and were recovered from all sources except from water and composted manure (Figure 8). As shown in figure 8, the majority of isolates were retrieved from raw chicken manure and litter samples. Isolates were predominantly recovered from farm 3. The ParSNP tree generated showed that seven isolates revived from raw chicken manure, produce, soil, and insect sources were closely related to each other. These isolates were collected from farm 3 and belonged to the phylogroup D, which is the largest represented phylogroup (n=14/255) detected in this subset. Interestingly, 93.4% of isolates possessed resistance genes to tellurite (Figure 6). Three different resistance profiles were identified: *terZABCDE* (n=205), *terABCD* (n=32), and *terBCD* (n=2) (Figure 6). Farm 5 was the most diverse and all three profiles were identified in isolates recovered from it.

3.6. Virulence Typing

Bioinformatics analysis of the MiSeq fastq sequence files of every *E. coli* isolate was performed to look for the presence of virulence genes: *stx1* (n=2/255), *stx2* (n=1/255), *eaeA* (n=29/255), *hlyA* (n=3/255), *bfpA* (n=0/255), *ipaH* (n=0/255), *elt* (n=0/255), *est* (n=0/255), *ehxA* (n=0/255), *aggR* (n=0/255), *aatA* (n=0/255), and *aaiC* (n=0/255). In total 31 *E. coli* isolates were able to be sorted into two pathotypes: enterohemorrhagic *E. coli* (EHEC, *stx* positive, n=3/255) and atypical enteropathogenic *E. coli* (aEPEC, *eae* positive and *stx* negative, n=28/255) as defined by Robins-Browne et al. 2016 and Pearson et al. 2016. All three EHEC isolates possessed genes, either *stx1* (n=2) or *stx2* (n=1). Two of these isolates (i.e., STEC 165, STEC 169) were collected from farm 5 in TN and were positive for *stx1*. Both isolates were sourced from raw manure either from a cattle pasture (STEC 169) or a shared sheep and goat pen (STEC 165). Likewise, isolate STEC 212 was sourced from cattle raw manure collected from farm 2 in NC and was positive for *stx2*. Isolates STEC 165 and STEC 212 were determined to be a part of phylogroup B1, while isolates STEC 169 was determined to belong to phylogroup B2 (Figure 6). Isolate STEC 165 was the only sample that contained all three virulence genes (*stx1*, *eaeA*, and *hlyA*). This profile is typically associated with STEC and exhibited a 100%, 89.18%, and 100% similarity, respectively, to the reference data in the virulence factor database. Both isolate STEC 169 (*stx1* and *hlyA*), and STEC 212 (*stx2* and *hlyA*) showed 100% similarity to their particular virulence genes.

All of the 28 aEPEC isolates were isolated from TN farms 3 (n=3/119) and 4 (n=25/65) and possessed *eaeA* (n=28/29) genes but did not have *bfpA*. The majority of aEPEC isolates that were MDR (n=17/28) belonged to the phylogroup E (n=12/28). However, 5 isolates from TN farm 4 were identified as phylogroup D. These isolates were all collected in June and July of

2015 and sourced from raw chicken manure, litter, and insect samples. Interestingly, all of these five isolates were characterized as MDR with a serotype of O167. They also possessed the sample tellurite resistance profile *ter*BCD. Of the three aEPEC isolates from TN farm 3, one was sourced from snap beans in July 2014; however, this isolate was not MDR.

4. Discussion

The primary objectives of this study were to 1) determine if the transmission of STEC or enteric pathogens was occurring on small and large animal herds or operations to fresh produce on sustainable farms in TN and NC, 2) identify the possible sources that impact transmission of AMR *E. coli*, specifically STEC on these systems, and 3) use WGS to characterize recovered *E. coli* from these sources. In April 2019 the Leafy Green Products Handler Marketing Agreement (LGMA) agency released an updated recommendation on the appropriate distance between the edge of a crop field and from composting operations (122 m) and CAFO's greater than 1,000 or 80,000 heads (366 m or 1610 m, respectively) (California Leafy Green Products Handler Marketing Agreement, 2019). The first aim of this study was to determine if transmission of STEC or enteric pathogens was occurring on sustainable farms in TN and NC and to determine if a 122 m distance between animal operations to the edge of produce fields was adequate to prevent the transmission of *E. coli*. Due to the significant size difference between TN (large scale production) and NC (small scale production) farms, it is essential to evaluate this aspect separately. NC farms 1 and 2 had at most 50 chickens and ten cows, respectively and were chosen because they maintained a distance between 15 m to 61 m from the edge of the crop to their chicken coop and cow pasture. Interestingly, no produce from either farm tested positive for *E. coli*. This is in direct contrast to the farms in TN, of which most of the poultry houses and cow

pastures were located between 3219 m to 9656 m away from the edge of the crop fields but had produce (cucumbers, snap beans, tomatoes, kohlrabi, melons, and cabbage) test positive for *E. coli*. With the above in mind, the data suggest that the transmission of *E. coli* on sustainable farms is occurring. However, the data suggest that the distance between CAFO's and the fresh produce field does not contribute to the transmission of *E. coli* from livestock to fresh produce. It seems that one of the most important factors could be the overall herd size on each farm.

The second objective was to identify the possible sources that impact the transmission of AMR *E. coli* on sustainable farms. Although there have been a few studies have attempted to identify the source of AMR bacteria isolated from fresh produce (Abakpa et al., 2015; Faour-Klingbeil et al., 2016; Holvoet et al., 2013; Marti et al., 2013), soil (Abakpa et al., 2015; Holvoet et al., 2013; Marti et al., 2013), manure (Abakpa et al., 2015; Marti et al., 2013; Tien et al., 2017), and insects (Alves et al., 2018; Mohammed et al., 2016; Ranjbar et al., 2016; Talley et al., 2009), none have investigated the interaction between all three potential sources (the environment, livestock, and insects) and its impact on the contamination of AMR bacteria on fresh produce concurrently. A few studies have focused on the role that insects, namely fruit and house flies, play in the transmission of foodborne pathogens onto fresh produce and fruit (Alves et al., 2018; Berry et al., 2015; Black et al., 2018; Sela et al., 2005; Talley et al., 2009). However, few have expanded that research to include AMR surveillance (Alves et al., 2018; Talley et al., 2009). A recent study conducted in Brazil, which characterized *E. coli* isolated from flies using PFGE and disk diffusion, reported that four MDR *E. coli* isolates from farms showed a 100% similarity to each other (Alves et al., 2018). Interestingly, a study conducted in 2015 revealed that 15-day old fruit flies could travel on average 2 a day (Chen et al., 2015). This may explain

why some produce samples tested positive for *E. coli* despite being located 1.98 miles away from the poultry houses on TN farms 3 and 4.

The World Health Organization and the FDA have released new guidelines that prohibit the use of medically important antibiotics for humans for growth promotion or feed efficiency in livestock (U.S. Food and Drug Administration, 2017; World Health Organization, 2017). In addition, the FDA's guidelines, Guidance for Industry (GFI) #213, required that the use of medically important antibiotics had to be done under the guidance of a licensed veterinarian (U.S. Food and Drug Administration, 2017). Currently the USDA National Organic Program (NOP) requires that raw manure must be applied to the soil at least 90 days from planting to harvest of the crop whose edible portions do not come in close contact with soil and at least 120 days from planting to harvest of the crop if the edible portions of the crop does come in contact with the soil (U.S. Department of Agriculture, National Center for Appropriate Technology, 2011; U.S. Department of Agriculture, National Organic Program, 2018). However, it is unknown if the 90 to 120-day interval is long enough to reduce the pathogen load in raw manure. A few studies have suggested that climate and soil type may be an essential factor when considering the survival time of *E. coli* in raw manure (Gutierrez-Rodriguez and Adhikari, 2018; Patterson et al., 2018; Sharma et al., 2016; Sharma et al., 2019). Similarly, studies have demonstrated that improperly composted manure mixed with soil can be a potential source of AMR pathogens in other studies (Abakpa et al., 2015; Marti et al., 2013; Tien et al., 2017). In this study, *E. coli* was primarily isolated from raw chicken manure, litter, and insect samples, as shown in figure 2. The results suggest that contaminated raw chicken manure, soil, and insects can be potential sources of AMR *E. coli* on fresh produce on sustainable farms. This is based off of figure 8, which shows that isolates collected from soil (239 and 240), produce (241 and 243),

and fly traps (245) on the same farm at the same time, share the same number and placement of SNP's in their core genome. This suggests that their core genome is similar to one another and it is probable that they come from the same source. Furthermore, on the next collection trip fecal samples from raw chicken manure (isolates 244 and 247) recovered *E. coli* isolates that had the same genetic fingerprint. However, it is important to note that the ParSNP tree is not a measure of evolutionary distance. Future experiments focused on the transmission of foodborne pathogens should include insect sampling into their methodology. It should also be noted that 16.3% of water samples (n=9/59) was positive for *E. coli* despite using municipal water for their operations. This is in agreement with a study conducted 2015 in Nigeria (Abakpa et al., 2015), which found that 18% of their irrigation water samples were contaminated with *Salmonella typhi*. However, this percentage is substantially lower than the percentages reported by Holvoet et al. (2013) and Decol et al. (2017), who reported that 59% and 84.8%, respectively, of the irrigation water samples collected was contaminated with *E. coli*.

The third objective of this study was to use WGS to characterize the serotype, AMR, and virulence profile of each isolate collected and to determine the phylogenetic relationship between each isolate. Of the top three serotypes identified in this study, only two were recovered in NC (O45, n=1/39; On12, n=11/39) from raw chicken manure. The lack of sero-diversity may be due to the small NC farm heard size was and the livestock diversity (dairy cattle and poultry farms) compared overall to the farms in TN (dairy cattle, poultry, sheep, goats, and pig farms). Based on WGS characterization, ten of the isolates were identified as serotype O45, which is a serotype that is commonly associated with the "Big Six" group and has been linked with foodborne illnesses in humans (U.S. Department of Agriculture, Food Safety and Inspection Service, 2012; U.S. Department of Health & Human Services, Centers for Disease Control and Prevention,

2006). Although these ten isolates do not possess *stx* genes it is possible for them to receive the gene from *stx*-converting phages (Hooton et al. 2019). Figure 5, revealed that NC farms had higher percentages of isolates that were resistant to antibiotics like ceftriaxone (AXO), ceftiofur (XNL), and cefoxitin (FOX), which are extended spectrum and AmpC beta-lactams respectively, than the TN farms. WGS also revealed that all NC isolates possessed *bla_{EC}* genes, a class C AmpC gene, which may explain the phenotypic resistance profile of these isolates. Only 35 isolates carried any of the targeted virulence genes and their variants. The all of NC isolates (n=39/39) were positive for virulence genes *eaeH* and *hlyE*. Isolate STEC 212 was positive for both *stx2A* and *stx2B* variants as well as *hly* variants A, B, C, D, and E. The majority of TN isolates were positive for virulence genes *eaeH* (n=179/196) and *hlyE* (n=196/216). Isolate STEC 165 was positive for both *stx1A* and *stx1B* variants as well as *hly* variants A, B, C, D, and E. Isolate STEC 169 was positive for both *stx1A* and *stx1B* variants as well as *hly* variants A, B, C, and D.

Isolates were also clustered into eight phylogroups (A, B1, B2, C, D, E, F, and G) based on their core genomes. This approach is similar to multi-locus sequence typing (MLST), and recent studies have revealed that certain phylogroups can be associated with different ecological niches and infections (Beghain et al., 2018; Clermont et al., 2015; Hutton et al., 2018). One-third of the isolates characterized were part of phylogroups B2 and D, which are more likely to be associated with extraintestinal infections in humans (Beghain et al., 2018; Van der Bij et al., 2011). The phylogroups A and B1, on the other hand, are considered to be universal and can be found in non-host environments like soil and water (Corzo-Ariyama et al., 2019). Interestingly, five out of nine *E. coli* isolates from water were MDR and represented phylogroups B1 (n=3), C (n=1), and D (n=4). *E. coli* isolates from NC farms belonged to phylogroups A (n=23/39), B1

(n=15/39), and D (n=1/39), while TN farms had isolates that belonged to all eight phylogroups tested (A, n=39/216; B1, n= 83/216; B2, n=5/216; C, n=10/216; D, n=46/216; E, n=14/216; F, n=3/216; and G, n=12/216). This suggests that the transmission of *E. coli* on NC farms may be due to the environmental transmission of *E. coli* through methods like wind or insect transmission. The high prevalence of phylogroups B2, D, and E suggest that there may be another source of transmission that has not been accounted for. This may be due to the farm and operation size of the TN farms and the diverse animal rearing occurring there. WGS also revealed that a large part of ESBL isolates identified as members of phylogroups B2 and D were MDR, which is concerning considering that they were collected from sustainable farms that do not use antimicrobials. Similar observations were reported from multiple studies from the Netherlands (Ranjan et al., 2017; Van Hoek et al., 2015). There is also growing evidence of *E. coli* securing novel AMR and virulence genes that can be located on transposons (Chowdhury et al., 2019) and plasmids (Carattoli et al., 2017; Liu et al., 2016; Xavier et al., 2016), which are then disseminated to other bacterial lineages and environments through horizontal gene transfer (Begum et al., 2018; Shen et al., 2018; Xavier et al., 2019). This may be the potential explanation of what is occurring on sustainable farms that we have visited, and it is concerning because *E. coli* is prevalent in many different environments.

We acknowledge that the overall number of farms involved in this study was small; TN (n=3/5) NC (n=2/5); however, each group represents an important section within the diversified farming community with small and large size operations that clearly have shown the differences in AMR and MDR within our analysis and within the distribution inside the farms. Further, we were able to show that transmission of *E. coli* between environmental samples (soil, raw manure, and insects) and fresh produce does happen on sustainable farms by demonstrating the similarity

of SNP number and placement of several isolates from various sources. The abundance of AMR genes and MDR *E. coli* on sustainable farms that do not use antimicrobials does raise some questions on what environmental factors are at play to select for these phenotypes. Therefore, a more in-depth and controlled analysis of the interactions between the environment, livestock, and fresh produce is needed in order to understand this observation fully.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Farm	Date Collected	Trip	Vegetables	Soil	Poultry Litter	Composted Manure	Raw Manure	Water	Insects	TOTAL Collected	Farm Totals
Farm 1	31/07/2014	1	65	15	5	20	20	0	11	136	487
Farm 1	25/08/2014	2	25	14	14	10	20	3	0	86	
Farm 1	13/10/2014	3	20	17	17	10	20	0	0	84	
Farm 1	31/03/2015	4	0	5	5	5	10	0	0	25	
Farm 1	01/06/2015	5	35	14	5	5	15	3	10	87	
Farm 1	24/08/2015	6	9	9	5	5	10	0	0	38	
Farm 1	13/09/2015	7	8	8	5	0	10	0	0	31	
Farm 2	16/12/2014	1	40	8	8	0	16	0	0	72	222
Farm 2	18/05/2015	2	25	5	10	0	10	1	5	56	
Farm 2	10/08/2015	3	12	10	12	0	10	0	10	54	
Farm 2	07/09/2015	4	10	10	10	0	10	0	0	40	
Farm 3	10/06/2014	1	40	18	0	0	2	3	2	65	549
Farm 3	25/06/2014	2	25	27	8	4	0	4	12	80	
Farm 3	14/07/2014	3	45	42	8	3	12	4	26	140	
Farm 3	01/08/2014	4	46	22	8	3	12	4	22	117	
Farm 3	31/05/2015	5	0	6	12	1	12	2	16	49	
Farm 3	03/06/2015	6	0	10	12	0	9	4	13	48	
Farm 3	27/07/2015	7	0	10	12	0	11	4	13	50	
Farm 4	23/06/2014	1	25	13	5	2	14	4	20	83	250
Farm 4	07/07/2014	2	18	15	9	1	13	4	25	85	
Farm 4	04/08/2014	3	20	12	8	1	14	3	24	82	
Farm 5	29/05/2015	1	10	12	0	0	9	4	10	45	151
Farm 5	15/06/2015	2	20	11	0	0	8	4	4	47	
Farm 5	13/07/2015	3	25	16	0	0	10	4	4	59	

Table 1. The total number of environmental samples collected between 2014 and 2015.

Figure Captions

FIGURE 1. Experimental Design of the project.

FIGURE 2. Distribution of *E. coli* isolates based on source type for each farm. States represented from left to right: North Carolina (NC) and Tennessee (TN). Total number of isolates collected from each farm from left to right: farm 1 (n=31), farm 2 (n=8), farm 3 (n=120), farm 4 (n=70), farm 5 (n=26). Total number of isolates collected from each source type from top to bottom: insect (n=30), water (n=9), soil (n=19), produce (n=14), manure (n=123), and poultry litter (n=60).

FIGURE 3. Prevalence of *E. coli* based on produce type. Only months with positive produce samples were shown for clarity. Total number of isolates collected each month from left to right: June 2014 (n=4), July 2014 (n=2), June 2015 (n=1), July 2015 (n=7). Total number of isolates per produce type from left to right: cucumber (n=6), kohlrabi (n=1), snap bean (n=3), melon (n=1), tomato (n=1), and red cabbage (n=2). Source type from left to right: poultry litter, raw manure, produce, soil, water, insect, and composted manure.

FIGURE 4. Prevalence of *E. coli* based on collected month and source type. Total number of isolates collected each month from left to right: March (n=3), May (n=7), June (n=93), July (n=92), August (n=47), September (n=11), December (n=2). Total number of isolates collected from each source type from left to right: poultry litter (n=60), raw manure (fecal, n=117), produce (n=14), soil (n=19), water (n=9), insect (n=30), and composted manure (n=6). Total number of isolates collected from each farm from left to right: farm 1 (n=31), farm 2 (n=8), farm 3 (n=120), farm 4 (n=70), farm 5 (n=26).

FIGURE 5. Percentages of *E. coli* that were determined resistant to the antibiotics tested based on broth microdilution. States represented from top to bottom: Tennessee (TN, black) and North Carolina (NC, grey). Antibiotics tested from left to right: ampicillin (AMP), Amoxicillin/clavulanic acid (AUG2), ceftriaxone (AXO), cefoxitin (FOX), ceftiofur (XNL), gentamicin (GEN), streptomycin (STR), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), chloramphenicol (CHL), and azithromycin (AZI).

FIGURE 6. ParSNP tree of *E. coli* isolates. Isolates were classified into seven phylogroups from top to bottom: B1, C, A, E, D, B2, and F. The presence (black stripes) or absence (white stripes) of housekeeping genes *arpA* (1), *chuA* (2), *yjaA* (3), and *tspE4* (4) is indicated in the second box. Chromosomal DNA was used to create the SNP tree. The visualization of the identified tellurite resistance profiles *terZABCDE* (n=205), *terABCD* (n=32), and *terBCD* (n=2) is in box three. Isolates that possessed STEC genes *stx1* (grey) and *stx2* (black) is contained in box four. Stripes in box four mark the isolates that belong the top three most prevalent serotypes identified in the study. From left to right: serotype O12 (n=11), O45 (n=10), O102 (n=19).

Figure 7. Number of isolates sorted by phylogroup and categorized by source. Total number identified by phylogroup from left to right: A (n=67), B1 (n=101), B2 (n=5), C (n=10), D (n=44), E (n=13), F (n=3) and G (n=12). Total number of isolates collected from each source type from top to bottom: water (n=9), soil (n=19), produce (n=14), composted manure (n=6), raw manure (n=117), poultry litter (n=60), and insects (n=30).

FIGURE 8. ESBL are defined as isolates that possessed the resistance genes *bla_{CMY}* and *bla_{TEM}*. ESBL isolates were classified into seven phylogroups (B1, C, A, E, D, B2,

and F). The chromosome DNA was used in order to create the SNP tree. The farm ID is depicted by the shape of the symbol next to each isolate while the color of each symbol represents the source type. ESBLs were only retrieved from Tennessee (TN) farms. Source categories from left to right: raw chicken manure (RCM), chicken litter, soil, produce, and insect.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8